Developmental Biology **223,** 205–215 (2000)
doi:10.1006/dbio.2000.9747, available online at http://www.idealibrary.com on IDEAL®

Induction of DNA Replication in the Germinal Vesicle of the Growing Mouse Oocyte

Renata Czołowska[1,2] and Ewa Borsuk[1]

Department of Embryology, Institute of Zoology, University of Warsaw, 00-927 Warsaw 64, Poland

Growing mouse oocytes are physiologically arrested in the G2 phase of prophase of the first meiotic division. Growing oocytes were isolated from ovaries of 9- to 12-day-old mice and fused with parthenogenetic one-cell eggs or two-cell embryos derived from fertilized eggs. Resulting hybrids were injected with Dig-11–dUTP and examined for DNA replication using immunofluorescence. Parthenogenetic one-cell eggs fused at telophase II, G1, and middle-to-late S phase, and also S-phase two-cell blastomeres, were able to trigger DNA synthesis in oocyte germinal vesicle (GV) in the majority of hybrids cultured to the end of the first cell cycle. Activation of replication in the GV occurred within 2–3 h after fusion of growing oocytes with S-phase eggs. We show indirectly that the reactivation of replication in GVs was not dependent on the breakdown of the GV envelope. Although GVs had the ability to renew DNA replication after fusion, the G2 blastomere nuclei were incapable of reinitiating DNA replication under the influence of S-phase one-cell eggs. We hypothesize that the nuclei of growing oocytes arrested in meiotic prophase are in a physiological state that is equivalent to replication-competent G1, and not G2, nuclei.

Key Words: **DNA replication; germinal vesicle; pronucleus; growing oocytes; parthenogenetic eggs; blastomeres; mouse; cell hybrids; transcription.**

INTRODUCTION

Most mitotically dividing eukaryotic cells replicate their DNA only once per cell cycle. It is believed that the activity of mitotic cyclin-dependent kinase(s) maintains G2 nuclei in the nonreplicating state and ensures that the replication of the genome is possible only after mitosis (reviewed by Nurse, 1997; see also Mahbubani *et al.*, 1997). The role of the nuclear envelope breakdown, the integral event of mitosis, in preparing (licensing) the chromatin for the new round of DNA replication was clearly demonstrated by Blow and Laskey (1988), who used a cell-free DNA replication system from activated *Xenopus* eggs. At the exit from mitosis, the activity of cyclin-dependent kinase (cdc2/cyclin B) drops and this allows the prereplication complexes (pre-RCs) to reassemble in early G1 nuclei (Mahbubani *et al.*, 1997; Hua and Newport, 1998). Pre-RCs are built by sequential binding of the chromatin proteins ORC, Cdc6, and Mcms (reviewed by DePamphilis, 1998). Binding of Mcms to chromatin (i.e., chromatin licensing) requires the activity of two replication licensing factors, RLF-B and RLF-M (*Xenopus;* Rowles *et al.*, 1999). These licensed pre-RCs are activated at the G1–S transition by diffusible S-phase-promoting factors: Cdk2/cyclins A and E (DePamphilis, 1998; Pasero and Gasser, 1998). DNA synthesis proceeds due to the activity of multiple enzymes of the replication machinery, like PCNA, RPA, polymerase α, and DNA ligase I (reviewed by Leonhardt and Cardoso, 1995).

The inhibition of $p34^{cdc2}$ kinase activity in G2 mammalian cells uncouples ordered events of the cell cycle and makes G2 nuclei competent to initiate a new round of DNA replication without an intervening mitosis. This was demonstrated in studies on fibroblasts exposed to protein kinase inhibitor K-252a (Usui *et al.*, 1991). The competence for DNA replication can also be restored in a cell-free system in which G2 nuclei of HeLa cells are treated with *Xenopus* S-phase extract after being preexposed to serine/threonine protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) (Coverly *et al.*, 1996, 1998).

Nuclei of ovarian oocytes of the mouse are arrested in the diplotene (dictyate) stage of meiotic prophase. They are

[1] Both authors contributed equally to the present work.

[2] To whom correspondence should be addressed. Department of Embryology, Institute of Zoology, University of Warsaw, Krakowshie Przedmieście 26/28, 00-927 Warsaw 64, Poland. Fax: (4822) 826 8624. E-mail: embrio@plearn.edu.pl.

0012-1606/00 $35.00

thought to be in a prolonged G2 phase because the last round of DNA replication occurred in the embryonic life, before the entry of germ cells into meiosis (Peters *et al.*, 1962; Lima-de-Faria *et al.*, 1962). To acquire the ability to renew DNA replication, germinal vesicle (GV) oocytes must grow, develop the competence to undergo maturation, pass through two meiotic divisions without an intervening S phase, and be activated. Activation (by sperm or artificial stimulus) initiates the first embryonic cell cycle with typical G1, S, and G2 phases. However, mechanisms that prevent ovarian oocytes from the initiation of DNA replication and mechanisms responsible for the initiation of DNA replication in fertilized (activated) eggs are poorly understood.

Preovulatory G2 mouse oocytes treated with 6-DMAP remain arrested in meiotic prophase (Szöllösi *et al.*, 1991), but do not initiate DNA replication (Fulka *et al.*, 1997). This shows that the G2 mechanisms which prevent DNA replication in somatic cells do not function in prophase oocytes. Reinitiation of DNA replication in oocyte nuclei was observed when preovulatory mouse oocytes were fused with recently activated parthenogenetic eggs cultured in the presence of 6-DMAP (Fulka *et al.*, 1997). However, the authors were uncertain whether the nuclear envelope of oocyte nuclei remained intact or was destabilized by the residual MPF activity present in recently activated parthenogenetic egg and/or in the hybrid cells. If fusions with egg cells were postponed for a few hours following activation, 6-DMAP was ineffective in inducing DNA synthesis in prophasic G2 germinal vesicle (Fulka *et al.*, 1997). These results remain inconclusive, since they do not discriminate between the effects produced by 6-DMAP and by the disintegration of the nuclear envelope. If the parthenogenetic eggs provide factors that induce DNA replication in G2 germinal vesicles, it is important to characterize the expression pattern of these factors during the first cell cycle of the parthenogenetic egg and to show how soon after fusion the GVs are capable of reacting to them.

To assay for DNA replication of prophase G2 nuclei under the influence of egg cytoplasm, in the absence of the kinase inhibitor 6-DMAP, we fused maturation-incompetent prophase G2 oocytes of the mouse with one-cell and two-cell embryos. We showed that the nuclei of prophase G2 oocytes are capable of undergoing DNA replication when subjected to the influence of cytoplasm of parthenogenetic one-cell eggs and two-cell embryos (derived from fertilized eggs) provided they had passed through the S phase of an embryonic cell cycle. We also demonstrated that in our system, induction of DNA replication in germinal vesicles proceeds rapidly and does not depend on destabilization of their envelope. Additionally, we provide evidence that meiotic G2 nuclei are unique in responding to the replication-activating environment, since G2 nuclei of two-cell stage blastomeres subjected to the same cytoplasmic signals do not initiate DNA replication. These findings suggest that the G2 nuclei of growing mouse oocytes that have completed DNA replication before entering meiosis are in fact in the physiological state corresponding to the replication-competent G1 nuclei.

MATERIALS AND METHODS

Growing ovarian oocytes, ovulated oocytes, and two-cell embryos were obtained from F1(C57Bl/10 × CBA/H) and F1(CBA/H × C57BL/10) mice. All chemicals were purchased from Sigma Co. unless stated otherwise.

Growing Oocytes

A uniform population of growing oocytes (diameter 50–60 μm) was obtained by puncturing the ovaries of sexually immature mice at 9–12 days postpartum. At this stage all oocytes are incompetent to mature spontaneously after isolation from the follicles. Maturation-competent oocytes are first observed on day 15 postpartum. Before fusion, oocytes were cultured from one to several hours in M2 medium supplemented with bovine serum albumin (BSA) (Fulton and Whittingham, 1978) under paraffin oil, at 37.5°C in 5% CO_2 in air. Zonae pellucidae were removed with 0.5% Pronase.

Ovulated Oocytes

Ovulated (metaphase II) oocytes were obtained from 2- to 5-month-old female mice induced to ovulate with pregnant mare's serum gonadotrophin (Intervet) and human chorionic gonadotrophin (hCG; Intervet) (doses: 10 IU of each given 45–52 h apart). Oocytes were harvested $16\frac{1}{2}$–18 h after hCG injection, treated with hyaluronidase (150 IU/ml) to remove cumulus cells, submitted to activation (see below), and cultured in M2+BSA as above. Zonae pellucidae were removed with 0.5% Pronase.

Activation

Ovulated oocytes at the age of 18–$18\frac{1}{2}$ h after hCG injection were activated by exposure to 8% ethanol for 7–8 min (Cuthbertson *et al.*, 1981; Cuthbertson, 1983) and cultured in M2+BSA as above.

Two-Cell Embryos

Two-cell stage embryos were obtained from superovulated females mated with F1 males. Females were autopsied 29 h (i.e., at the time when the first two-cell embryos can be recovered) and 39–41 h (advanced two-cell embryos) after hCG injection. After removal of zonae pellucidae, two-cell embryos were cultured in M2 medium under standard conditions until used for fusion.

Fusion

Activated oocytes (parthenogenetic eggs), two-cell embryos, or single blastomeres and growing oocytes were preincubated for a few minutes in phytohemagglutinin (300 μg/ml in BSA-free M2 medium) in an agar-coated embryological watch glass and agglutinated into pairs (parthenogenetic egg + growing oocyte or two-cell blastomere + growing oocyte or two-cell blastomere + parthenogenetic egg). Pairs were washed twice in 0.25 M glucose supplemented with 100 μM $CaCl_2 \cdot 2H_2O$ and 100 μM $MgSO_4 \cdot 7H_2O$ and

transferred to a fusion chamber filled with the same solution. After the couplet had been correctly oriented between the platinum electrodes (electrode gap 116 μm), four 40-V pulses of 25 μs duration were applied. The couplet was then thoroughly washed in M2 medium and cultured under standard conditions. Time of fusion was recorded for individual pairs. Fusion usually occurred within 1–2 h of electric pulse application, though some pairs needed a much longer time to fuse. Hybrids were injected with precursors of DNA replication or transcription (see below) and cultured in M2 medium.

To block DNA synthesis in parthenogenetic one-cell eggs (cultured with the inhibitor from the moment of activation) and in the resulting hybrids, the M2 medium was supplemented with aphidicolin (3 μg/ml) in one series of control experiments.

Each fusion experiment was performed in several series. The age (expressed in hours after activation with ethanol) of parthenogenetic eggs at the time of fusion with growing oocytes was correlated with cell cycle phase, as follows: 2–3 h (telophase II, G1), 5–7 h (G1, S), $7\frac{1}{2}$–$9\frac{1}{2}$ h (S), $7\frac{1}{2}$–11 h (S, G2).

In those experiments in which two-cell embryos were used for fusion, their age at the moment of fusion was 31–33 h post-hCG (phase G1–S and S) or 44–45 h post-hCG (G2 phase).

Detection of DNA Replication

Cell hybrids and control cells were microinjected into the cytoplasm with a precursor of DNA replication, digoxigenin-11–dUTP (Dig-11–dUTP; 1 mM solution—undiluted commercial product; Boehringer), using an Eppendorf microinjector (Bouniol-Baly *et al.*, 1997). The injected volume was always less than 2 pl (ca. 1% of the volume of the parthenogenetic egg). Hybrids were injected at different time intervals after fusion, ranging from a few minutes to 4 h. Control cells (parthenogenetic pronuclear eggs, two-cell embryos, and growing oocytes) were injected like the hybrids. After the injection, hybrids and controls were cultured for different periods of time (see Results), and then they were fixed for immunofluorescence according to Bouniol *et al.* (1995). Dig-11–dUTP incorporation into newly synthesized DNA was detected by indirect immunofluorescence using mouse monoclonal anti-digoxigenin primary antibody (Boehringer) and an FITC-conjugated goat anti-mouse IgG (H+L) secondary antibody (Caltag Laboratories). To identify nuclei, hybrids and controls were stained with a chromatin-specific dye, Hoechst 33342 (2–4 μg/ml PBS), following incubation with secondary antibody. The specificity of the immunoreaction was checked in parthenogenetic eggs injected with the Dig-11–dUTP and stained without the primary antibody treatment. Hybrids and controls were analyzed using an epifluorescence microscope (Axiovert 135; Zeiss). Images were captured with a Variocam (PCO) CCD camera. Some hybrids were examined with a Zeiss LSM 510 confocal microscope.

Detection of Transcription

Transcription in hybrid cells and controls (isolated growing oocytes) was examined according to the method of Bouniol *et al.* (1995). Hybrids and oocytes were microinjected with 1–2 pl of BrUTP (Sigma; 100 mM solution in 2 mM Pipes buffer with 140 mM KCl, pH 7.4) and cultured in M2 medium. Fixation and permeabilization were done as described above. BrU incorporation was detected by indirect immunofluorescence using mouse monoclonal anti-BrdU antibody (IgG) (Caltag Laboratories) as the primary antibody and FITC-conjugated goat anti-mouse IgG (H+L) (Caltag Laboratories) or a rhodamine-conjugated donkey anti-mouse IgG (H+L) (Jackson Immunoresearch) as a secondary antibody. Following incubation with the secondary antibody nuclei were stained with Hoechst 33342. Preparations were analyzed using an epifluorescence microscope (Axiovert 135; Zeiss). Images were captured with a Variocam (PCO) CCD camera.

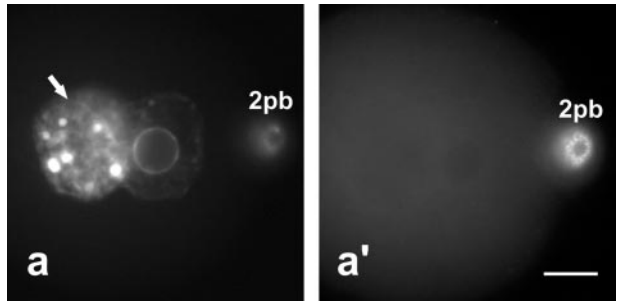

FIG. 1. A hybrid cell formed between a parthenogenetic egg in the postreplicative (G2) stage and a growing oocyte from prepubertal mouse. GV indicated by arrow. (a) Morphology of the pronucleus and the GV. DNA stained with Hoechst 33342. (a′) Neither nucleus shows incorporation of Dig-11–dUTP and thus both are inactive in DNA replication. Nucleus of the second polar body (2pb) is active in DNA replication. Bar, 20 μm.

RESULTS

First Embryonic Cell Cycle of Parthenogenetic Eggs

After the completion of second meiotic division and extrusion of the second polar body, activated mouse eggs enter interphase with G1, S, and G2 phases. The length of the first cell cycle and the length of individual phases of the cell cycle depend on the postovulatory age of oocytes at the time of activation. Oocytes of F1 mice activated with ethanol 21 h after hCG injection have an 18-h cell cycle with G1 5 h, S 6 h, and G2+M 7 h (Severova *et al.*, 1989). In our studies we used F1 oocytes activated 18 h after hCG injection. These parthenogenetic eggs enter S phase about 5–6 h after activation (aa) and complete replication about 13 h aa (unpublished results; Borsuk *et al.*, in preparation).

DNA Replication in Hybrids Formed between Parthenogenetic Eggs and Growing Oocytes

In a hybrid cell, the pronucleus (PN) can be distinguished from the GV on the basis of its morphology as visualized by staining of the chromatin with a fluorescent dye, Hoechst 33342. The chromatin of PN (1C DNA) is highly decondensed. The chromatin threads are very thin and uniformly dispersed, which makes the PN less bright than the GV. An easily visible nucleolus-like body is often surrounded by a bright ring of heterochromatin (Figs. 1a and 2a–2d). The chromatin of the GV is much more dense (4C DNA) and mostly homogeneous with only a few bright spots of

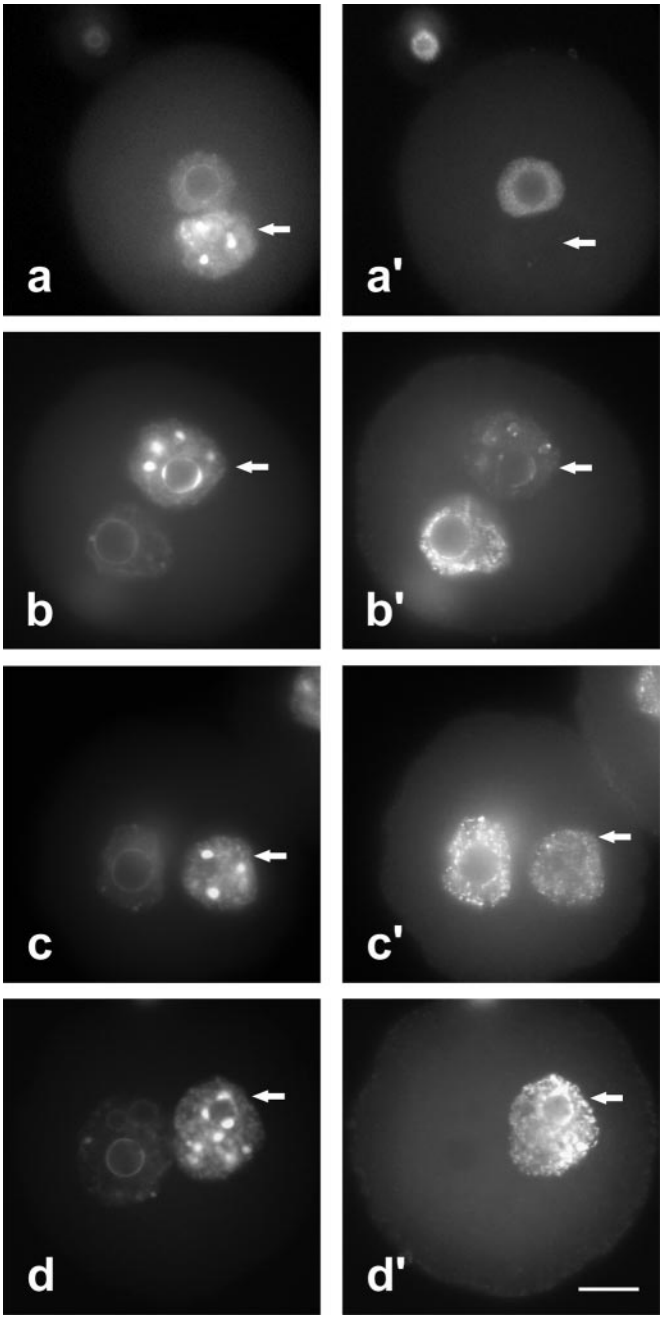

FIG. 2. Patterns of DNA replication in pronuclei and germinal vesicles in hybrids formed between parthenogenetic pronuclear eggs and growing oocytes. Hybrids were cultured 22–26 h after egg activation. Arrows indicate GVs. (a–d) DNA stained with Hoechst 33342. (a′–d′) Immunofluorescent detection of Dig-11–dUTP incorporated into newly synthesized DNA. (a and a′) The pronucleus and the second polar body nucleus are active in replication, GV does not replicate; (b and b′) the pronucleus is replicating, the GV initiates replication (the number of labeled replication sites is limited, some of them are localized in the proximity of more condensed chromatin); (c and c′) both the pronucleus and the GV are actively replicating, the intensity of labeling and the number of replication sites in both nuclei are comparable; (d and d′) the pronucleus does not replicate, the GV replicates (note high intensity signal over GV). Bar, 20 μm.

heterochromatin. These spots are often located in the proximity of nucleoli (one or two) which are distinguishable as areas of low Hoechst fluorescence (Figs. 1a and 2a–2d).

Table 1 summarizes results of DNA replication from 126 hybrids submitted to long and short culture. Hybrids were divided into five classes depending on immunoreactivity of the PN versus the GV. The advancement of the nuclei in DNA replication was estimated according to the criteria used by Ferreira and Carmo-Fonseca (1997) and Bouniol *et al.* (1997) for the pronuclei in the zygotes.

1. PN negative/GV negative: No DNA replication in either nucleus (Fig. 1b).
2. PN positive/GV negative: PN initiates DNA replication or it is in an advanced S phase, GV does not replicate (Fig. 2a′).
3. PN positive/GV initiating: PN is in an advanced stage of DNA replication, GV initiates replication (bright spots scattered in the nucleus) (Fig. 2b′).
4. PN positive/GV positive: PN is in an advanced stage of DNA replication or approaching the terminal phase of DNA replication (only heterochromatic regions are stained). GV is in early or advanced stages of DNA replication (Fig. 2c′).
5. PN negative/GV positive: PN does not replicate, GV is in an advanced stage of replication (Fig. 2d′).

In hybrids formed at the beginning of the egg cell cycle (telophase II and early G1, i.e., between 2 and 3 h aa, Table 1) the germinal vesicle competes for nuclear proteins with the egg nucleus leading to the suppression of pronuclear development and causing significant swelling of the GV (see also Fulka *et al.*, 1996). This was particularly evident in three hybrids showing a highly compact and nonreplicating pronuclei beside the enlarged GVs (Figs. 3a, 3b, and 3b′). In the remaining hybrids pronuclei were in different stages of decondensation and all were replicating. Germinal vesicles were replicating DNA in all 17 hybrids, and in 5 of them the staining reaction was very strong (Fig. 3a′). These results show that when the hybrids were formed in telophase II or G1 stages, the GVs were capable of participation in the egg cell cycle and were able to enter the S phase.

In hybrids produced between 5 and 7 h aa (G1–S phase transition and early S phase of an activated egg) the replicating pronuclei were recorded in 95% of the cases (53/56; Table 1). Replicating germinal vesicles were found in only 34% of the hybrids. The staining pattern suggested that DNA synthesis in GVs did not proceed beyond the phase of initiation although accompanying egg pronuclei were always well advanced in DNA synthesis as shown in Fig. 2b′. These results suggest that the GVs exposed to the egg cytoplasm at about the onset of S phase may have difficulties in crossing the G1/S border and are less reactive to S-phase egg factors.

TABLE 1
DNA Replication in Pronuclei (PN) and Germinal Vesicles (GVs) of Hybrid Cells

Age of egg at fusion, h aa (phase of cycle)	Time of hybrid culture (h aa)	Replicating nuclei (number of hybrids)					Number of hybrids with replicating GV (%)
		PN: −	+	+	+	−	
		GV: −	−	→	+	+	
2–3 (TII, G1)	25			6	8	3	17/17 (100)
5–7 (G1, S)	22	3	34 [4]	18	1		19/56 (34)
$7\frac{1}{2}$–11 (S, G2)	25–26	3	1 [1]	10	24 [3]	4	38/42 (90)
$7\frac{1}{2}$–$9\frac{1}{2}$ (S)	$10\frac{1}{2}$–$11\frac{1}{2}$		1	10			10/11 (91)

Note. aa—after activation. Nuclei: replicating (+), nonreplicating (−), initiating replication (→). Numbers in square brackets represent the numbers of hybrids in mitosis in which the origin of metaphase chromosomes (pronucleus versus GV derived) could have been identified.

The next experimental group consisted of hybrids which were produced from GV oocytes and PN eggs between $7\frac{1}{2}$ and 11 h after activation, i.e., during, and at the end of, S phase (Table 1). In most of those hybrids two replicating nuclei of diverse origin were observed (Fig. 2c′). Replicating germinal vesicles were detected in 90% (38/42) of hybrids. Most of these hybrids had GVs in an advanced stage of DNA replication (24/42), and those in which the GV just initiated replication were in the minority (10/42). Three hybrids showed both the PN and the GV as being negative (Fig. 1). These might have been the hybrids that did not enter S phase or those in which the pronucleate partner was in G2 phase at the time of fusion. In 4 hybrids, a nonreplicating pronucleus and a replicating GV were observed (Fig. 2d′). In these hybrids pronucleate eggs might have already terminated replication by the time of fusion but could still contain factors promoting DNA synthesis. In summary these results may indicate that the germinal vesicle is prone to react to S-phase inducers present between the middle and the end of S phase of the first cell cycle of the parthenogenetic egg.

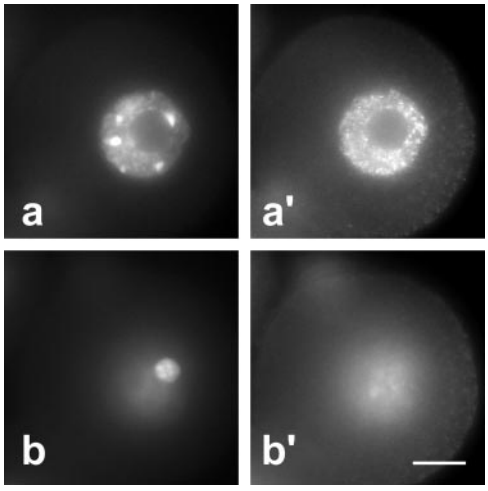

FIG. 3. DNA replication in the germinal vesicle in the hybrid between oocyte and parthenogenetic egg 2–3 h after its activation. Hybrids cultured 25 h after egg activation. (a and b) DNA stained with Hoechst 33342. (a′ and b′) Immunofluorescent detection of Dig-11–dUTP incorporated into newly synthesized DNA. An actively replicating GV (a and a′) coexists with a compact, nonreplicating egg pronucleus (b and b′). Bar, 20 μm.

The specificity of the immunostaining reaction was confirmed in control experiments in which DNA synthesis was blocked by culturing recently activated parthenogenetic eggs and hybrid cells (fusions between 7 and 8 h aa) in the presence of aphidicolin for 25 h. Whereas in all five aphidicolin-treated hybrids both nuclei were unstained, in all five hybrids cultured in pure M2 medium both nuclei were positively stained. A lack of staining was also recorded in parthenogenetic eggs injected with Dig-11–dUTP in S phase (9 h aa) and cultured under standard conditions but stained without incubation with the primary antibody.

Isolated growing oocytes injected with the precursor of DNA replication served as the additional control group. This group consisted of 17 injected oocytes cultured for 22 h and 30 oocytes which were submitted, before injection, to the same treatment that was used for the fusion (including electric pulses) and cultured 6–7 h after pulse application. None of these control oocytes showed a positive reaction for DNA replication. We thus rejected the possibility that the growing oocytes might have been engaged in DNA repair synthesis. DNA repair synthesis occurs in mouse oocytes at the pachytene stage (Moses *et al.*, 1984; Baker *et al.*, 1996) and was also detected in fully grown oocytes of *Xenopus* (Furuno *et al.*, 1994).

From this series of experiments we concluded that the germinal vesicle of the growing oocyte is capable of initiating DNA synthesis under the influence of a factor(s) present in the parthenogenetic egg.

GV Initiates Replication Soon after Fusion with the S-Phase Parthenogenetic One-Cell Egg

To learn how soon the germinal vesicle becomes capable of initiating replication after fusion with parthenogenetic

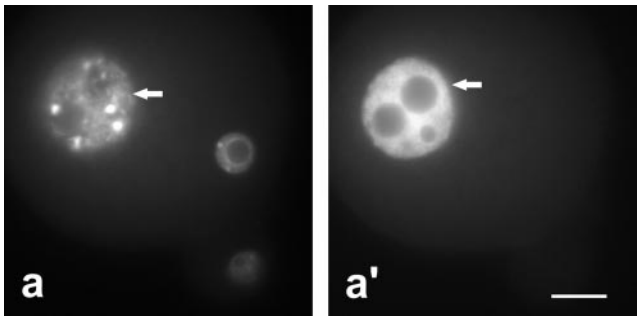

FIG. 4. RNA synthesis in nuclei of a hybrid cell formed between a parthenogenetic egg and a growing oocyte 2 h after activation and cultured 4 h after fusion. (a) DNA stained with Hoechst 33342. (a′) Immunofluorescent detection of BrU incorporation into nascent RNA. Actively transcribing GV (arrow) is visible beside the small, nontranscribing pronucleus. Bar, 20 μm.

egg active in DNA synthesis, we produced hybrids between $7\frac{1}{2}$ and $9\frac{1}{2}$ h after activation. Hybrids were injected with Dig-11–dUTP between 30 min and 2 h after fusion, and they were cultured for up to $10\frac{1}{2}$–$11\frac{1}{2}$ h aa (about 2–3 h after fusion). All but 1 of 11 hybrids from this series showed the presence of the GV initiating replication beside the pronucleus that was in an advanced stage of replication (Table 1). This indicates that in the S-phase egg, the GV is capable of initiating DNA replication within the first 2–3 h following fusion.

The GV Continues Transcription after Fusion with the Parthenogenetic One-Cell Egg

This series of experiments was performed to prove that germinal vesicles remain intact in hybrid cells and that the activation of DNA replication in germinal vesicles does not depend on a transitory breakdown of the nuclear envelope caused by a cytoplasmic factor(s) present in hybrid cytoplasm (see Introduction and Discussion). It has been shown that the nuclei of growing mouse oocytes exhibit high transcriptional activity (Bouniol-Baly *et al.*, 1999). In parthenogenetic eggs activated at 18 h after hCG injection transcription starts about 10–11 h aa (Borsuk *et al.*, in preparation). It has also been shown that the transcribing nuclei of exogenous origin which undergo temporary nuclear envelope breakdown in parthenogenetic mouse eggs stop the transcription (Borsuk *et al.*, 1996; Szöllösi *et al.*, 1998). We expected that if there is any destabilization of the GV envelope this would lead to the termination of the transcription in the germinal vesicle in our hybrids. To address this question we injected BrUTP—the precursor of RNA synthesis—into the hybrids between GV oocytes and parthenogenetic eggs formed early (about 2 h) and late (6–8 h) after egg activation. Subsequently the hybrids were cultured 8–9 h aa. In 10 of 11 hybrids from early fusions and in all 9 hybrids from late fusions, pronuclei were always unstained, whereas oocyte nuclei displayed a clear staining reaction (Fig. 4) (controls: 11 isolated growing oocytes, all GVs positively stained). In only 1 of 11 hybrids the GV was very weakly stained. These experiments suggest that the nuclear envelope of GVs remained intact and this leads to the conclusion that the breakdown of the nuclear envelope was not involved in the mechanism of activation of replication in the GV introduced into an activated egg.

Entry of Hybrids into the First Mitosis

We observed that some of the hybrids used for DNA replication experiments entered into the first mitosis (Table 1; Fig. 5). Since we expected that hybrids injected with the precursor of DNA synthesis may have difficulties entering into mitosis, we also examined whole-mount preparations of uninjected hybrids (which were processed according to Tarkowski and Wróblewska, 1967). Table 2 shows that none of the hybrids produced from GV oocytes and parthenogenetic eggs 1 to 3 h aa, as well as 5–7 h aa, have entered mitosis even after 49 h culture. In the control group, 90–100% of parthenogenotes divided or were blocked in mitosis. However, 50% of hybrids formed 8–9 h aa entered mitosis. In this control group 100% of parthenogenetic eggs divided. This suggests that only those hybrids in which the egg-cell component was in the advanced S phase at the time of fusion were capable of proceeding to M phase.

GV Replicates DNA in Two-Cell Embryos

To address the question whether DNA replication in the germinal vesicle could also be triggered by an S-phase blastomere of a cleaving embryo, we fused S-phase two-cell embryos derived from fertilized eggs with growing oocytes. In this experiment oocytes have usually fused with both blastomeres of two-cell embryos (14/15 hybrids). Embryos

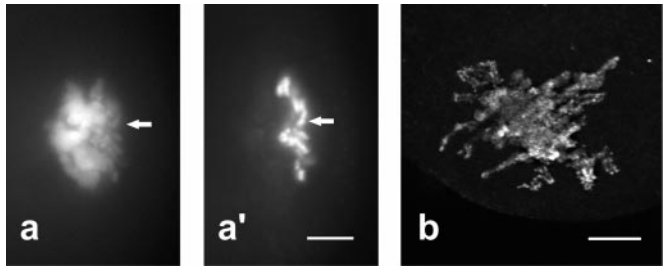

FIG. 5. Two hybrid cells formed between early (a and a′) and late (b) parthenogenetic S-phase eggs and growing oocytes, at the first mitosis. Pictures taken from: (a, a′) epifluorescence microscope, (b) laser scanning confocal microscope. (a) A group of chromosomes derived from pronucleus and GV, stained with Hoechst 33342. (a′) Incorporation of Dig-11–dUTP is visible only in chromosomes of pronuclear origin (arrow). Bar, 20 μm; (b) Both the pronucleus and the GV-derived chromosomes show incorporation of Dig-11–dUTP. Bar, 20 μm.

TABLE 2
Entry of Hybrids between Parthenogenetic Eggs and Growing Oocytes into Mitosis

Time of fusion, h aa (phase of cycle)	Time of hybrid culture (h aa)	Number of hybrids in		Number of controls in[a]	
		Interphase	Mitosis	Interphase	Mitosis
$1\frac{1}{2}$–$3\frac{1}{4}$ (TII, G1)	20–29	26		2	19
$5\frac{1}{2}$–$6\frac{3}{4}$ (G1, S)	49	16			18
8–9 (S)	$23\frac{1}{2}$	10	10		4

Note. aa—after activation.
[a] Single parthenogenetic one-cell eggs and fused pairs of two parthenogenetic one-cell eggs.

fused at the beginning of the second cell cycle (31–33 h after hCG injection) were expected to be at G1–S transition or in S phase (Płusa *et al.*, 1997). As shown in Table 3, in the majority of hybrids (13/15) cultured for 16–18 h both the blastomere nucleus(ei) and the germinal vesicle were replicating (Fig. 6). These results show that the GV from the growing oocyte is capable of replicating DNA under the influence of the factor(s) present in two-cell mouse embryos.

G2 Blastomere Nucleus Is Unable to Initiate DNA Replication in One-Cell Parthenogenetic Egg

Embryos of our F1 mice enter the second mitotic division at 47–49 h post-hCG injection (Tarkowski *et al.*, 1977). We have used single two-cell stage blastomeres at the age of 44–45 h post-hCG injection for the fusion. The control group consisted of 14 two-cell embryos. All of them were in G2 phase of the second cell cycle and did not replicate DNA. In the hybrids between such blastomeres and S-phase parthenogenetic eggs, none of the blastomere nuclei showed positive reaction for DNA replication after 26 h of culture (Table 4; Fig. 7). We conclude that G2 blastomere nuclei were incompetent to initiate a new round of DNA replication under the influence of the factors present in parthenogenetic one-cell eggs.

DISCUSSION

Cell fusion experiments with somatic cells have shown that S-phase cells contain activators of DNA replication which act on G1 but not on G2 nuclei (Rao and Johnson, 1970). In the present work we used a hybrid system consisting of embryonic cells: one- and two-cell embryos and growing oocytes. We demonstrated that the nucleus of a growing oocyte, which is thought to be arrested in G2 phase, is able to replicate DNA under the influence of S-phase factors present in one- and two-cell embryos. This finding is intriguing in the light of recent reports on experimental induction of DNA synthesis in G2 nuclei (Pasero and Gasser, 1998; see also Introduction for references). To our knowledge, our work demonstrates for the first time the inductive influence of the S-phase cell on the intact postreplicative nucleus.

In our study DNA replication in germinal vesicles was activated within 2–3 h after fusion with pronuclear S-phase parthenogenetic eggs, and the replication intensified with the time of hybrid culture. Eggs advanced in S phase at the moment of fusion and those terminating DNA replication were able to trigger DNA replication in GV in most of hybrid cells. Fusion of GV oocytes with telophase II and G1 parthenogenetic eggs was equally effective provided that GVs passed through the S phase of the "host" egg. Surprisingly, eggs around the onset of S phase were poor inducers

TABLE 3
DNA Replication in Blastomere Nuclei (BL) and Germinal Vesicles (GVs) of Hybrid Cells

Blastomere fusion, h post-hCG (phase of cycle)	Time of hybrid culture, h post-hCG (h postfusion)	Replication in the nuclei (number of hybrids)			Hybrids with replicating GV
		BL: +	+	+	
		GV: −	→	+	No. (%)
31–33 (G1, S)	49(16–18)	2	3	10	13/15(87)

Note. Nuclei: replicating (+), nonreplicating (−), initiating replication (→).

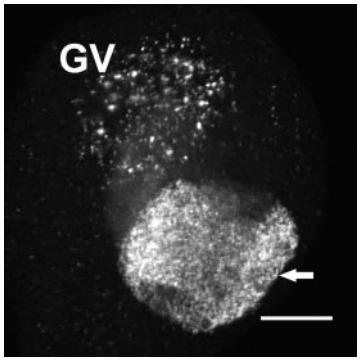

FIG. 6. Hybrid between S-phase two-cell embryo and a growing oocyte. Both the germinal vesicle (GV) and the blastomere nucleus (arrow) are actively replicating (laser scanning confocal microscope). Bar, 20 μm.

of replication: they were able to induce DNA replication in GVs in only one-third of hybrids. In addition these nuclei never progressed beyond the earliest stage of DNA replication. We suggest that at this particular stage both nuclei of the hybrid cell (pronucleus and GV) compete for positively acting S-phase factors which are rapidly used by the native egg pronucleus. Alternatively, growing oocytes may contain factors inhibiting replication of DNA in the GV which cannot be abrogated under this particular fusion protocol. Presence of negative regulators of DNA synthesis that affect initiation of replication (formation of active replication foci) was demonstrated in immature *Xenopus* oocytes (Zhao and Benbow, 1994; Fang and Benbow, 1996).

In proliferating cells the checkpoint control for mitosis ensures that the nucleus must complete S phase before a cell is allowed to enter mitosis (Hartwell and Weinert, 1989; reviewed by Nurse, 1997). Since some hybrids injected with Dig-11–dUTP entered mitosis, we analyzed conditions which facilitated progression of heterokaryons to the end of the cell cycle. In mitotic hybrids originating from early S-phase parthenogenetic eggs and growing oocytes only chromosomes of the pronuclear origin were immunostained. This suggested that the egg pronucleus has passed through the normal round of DNA replication, whereas the GV had not replicated. Mitosis was permitted because DNA in both nuclei was duplicated. In mitotic hybrids formed from late S-phase eggs all metaphase chromosomes were stained; DNA replication might have been completed in both nuclei, and mitosis of the postreplicative cell was permitted. The fact that 50% of hybrids formed from late S-phase eggs (which were not injected with Dig-11–dUTP) entered M phase confirms the above interpretation. In contrast, none of the hybrids formed from parthenogenetic eggs between telophase II and early S phase were capable of approaching mitosis. Either the replication of a pronucleus was disturbed (suppressed development of a pronucleus) or the replication of the germinal vesicle was not completed or both. All these conclusions are based on an indirect approach and need to be verified by measuring DNA content of nuclei in heterokaryons.

In the somatic cell cycle, nuclear envelope breakdown (NEBD) in mitosis is required to convert a postreplicative G2 nucleus into a nucleus licensed to build new prereplication complexes (Thömmes and Blow, 1997). In our hybrids, reinitiation of DNA replication in the germinal vesicle occurred without the breakdown of the nuclear envelope. We have indirectly excluded the possibility of NEBD of germinal vesicle by analyzing transcriptional activity of GVs after their transfer into parthenogenetic one-cell eggs. Nuclei which undergo NEBD after introduction into newly activated eggs (60–90 min aa) immediately terminate RNA synthesis and remain transcriptionally inactive until late S phase of the first cell cycle. In contrast, nuclei which do not undergo NEBD remain transcriptionally active (Borsuk *et al.*, 1996; Szöllösi *et al.*, 1998; Borsuk and Maleszewski, in preparation). Since in our hybrids the oocyte nuclei did not stop RNA synthesis, neither following fusion with recently activated eggs nor after the fusion with pronucleate eggs, we concluded that their nuclear envelope remained intact. Therefore we conclude that in our system the activation of DNA replication in the germinal vesicle depends on a mechanism(s) different from that operating in mitosis.

Since mammalian oocytes spend the greatest part of ovarian life in meiotic arrest (diplotene of meiotic prophase), they must have developed protective mechanisms against premature entry into M phase (meiotic matu-

TABLE 4

DNA Replication in Pronuclei (PN) and Blastomere Nuclei (BL) of Hybrid Cells

Age of eggs at fusion, h aa (phase of cycle)	Age of blastomeres at fusion, h post-hCG (phase of cycle)	Time of hybrids culture, h aa	Total number of hybrids	Replication of nuclei, No. (%) of hybrids		
				PN: +	+	−
				BL: −	+	−
7–8 (S)	44–45(G2)	26	17	15(88)		2(12)

Note. aa—after activation. Nuclei: replicating (+), nonreplicating (−).

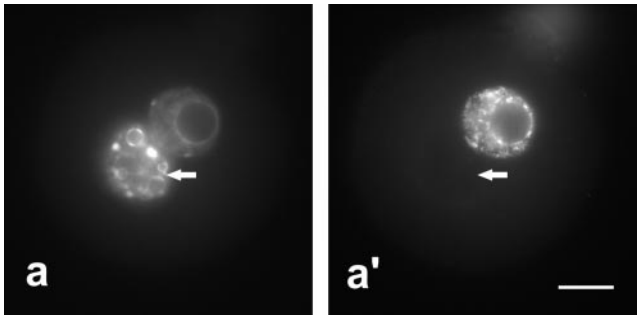

FIG. 7. DNA replication in a hybrid cell formed between an S-phase parthenogenetic egg and a G2 blastomere of a two-cell embryo. (a) Nuclei visualized with Hoechst 33342. (a′) Immunofluorescent detection of DNA replication shows replicating egg pronucleus and a nonreplicating blastomere nucleus (arrow). Bar, 20 μm.

ration), as well as undesired DNA replication. Maturation-competent mouse oocytes are maintained in meiotic arrest via the cAMP/protein kinase A pathway in which phosphorylation of $p34^{cdc2}$ prevents MPF activation (reviewed in Dekel, 1995). The cAMP-dependent mechanism, however, does not play a role in the meiotic arrest of maturation-incompetent growing oocytes (Bornslaeger *et al.*, 1988). Oocytes remain in G2 arrest because they are deficient in MPF components ($p34^{cdc2}$ and cyclin B) or are lacking the factors that trigger MPF activation or both (Chesnel and Eppig, 1995; de Vantery *et al.*, 1997). So far, the question how the maturation-incompetent mouse oocytes are protected against DNA rereplication has received little attention. The mechanism of G2-specific, $p34^{cdc2}$/cyclin B kinase which blocks assembly of new prereplication complexes in proliferating cells is not present in growing oocytes. This conclusion finds additional support in our experiments: G2 nuclei of proliferating two-cell embryos were resistant to S-phase-promoting factors provided by parthenogenetic egg cells (as was shown earlier by Fulka *et al.*, 1996), whereas G2 germinal vesicles of growing oocytes readily reactivated replication in one-cell eggs and two-cell embryos. These facts suggest that DNA replication in G2 growing mouse oocytes is blocked by a mechanism unique to the meiotic cells and that this block can be easily overridden by fusion with proliferating embryonic cells. Unfortunately, there are no studies on mammalian oocytes which might contribute to the understanding of how this DNA replication block is brought about. In their recent work Nakajo *et al.* (1999) gave convincing evidence that Chk1 kinase (checkpoint kinase 1), which is involved in the DNA damage G2 checkpoint, functions also in ovarian G2-arrested *Xenopus* oocytes. Chk1 was shown to localize to meiotic chromosomes in late zygotene/pachytene spermatocytes of the mouse (Flaggs *et al.*, 1997), but its possible function in the regulation of DNA replication and G2 phase of meiotic mouse oocytes remains to be proven.

In our experiments, the GVs of growing mouse oocytes readily reactivated DNA synthesis after fusion with S-phase one-cell eggs and two-cell embryos. Since this occurred in the absence of any kinase inhibitor, and without the intervening nuclear envelope breakdown, we believe that the nuclei of growing oocytes are in the physiological state corresponding to G1 rather than to G2 phase. This indicates that these nuclei may already be licensed for DNA replication, having prereplication complexes in the initiation-competent state (see De Pamphilis, 1998). What may be missing in a growing oocyte are the activators of DNA replication (cdk2/cyclins A and E). These in our system are supplied by the S-phase embryonic cells. Transcripts for G1–S cyclins and cdks are present in maturation-incompetent mouse oocytes but are not translated before oocyte maturation (Moore *et al.*, 1996).

In summary, our work provides the first experimental evidence that G2 nuclei of growing oocytes of mammals are different from G2 nuclei of proliferating cells. If the oocyte chromatin is already licensed for DNA replication, we should revise our view of the meiotic cycle, with classic G1, S, G2, and M phases. The competence for DNA replication may be acquired by oocyte chromatin at the early meiotic prophase (leptotene to early zygotene stages) when the oocyte chromosomes pass through the condensation/recondensation cycle during meiotic recombination. This phase of meiosis may be equivalent to M phase of the somatic cell cycle (though NEBD and karyokinesis do not occur), thus creating the window within which the prereplication complexes may be built. The competence for DNA replication is hidden in the intact growing oocyte, either because of lack of activators or because of presence of inhibitors of replication, but can be revealed in this experimental system.

Significant progress in the understanding of how the replication complexes are built and activated in metazoan cells has been based on cell-free systems of extracts from ovarian oocytes and parthenogenetic eggs of *Xenopus* and isolated embryonic and somatic nuclei. A corresponding cell-free system in mammals does not exist. The experimental system used in the present work offers the means to study various aspects of DNA replication at the cellular level and may allow the identification of proteins engaged in the control of DNA replication and G2 arrest in meiotic mammalian cells.

ACKNOWLEDGMENTS

This work was financed by a grant from the State Committee for Scientific Research (Grant 6PO4C 048 15). The authors thank Professor A. K. Tarkowski for his valuable suggestions and comments on the manuscript and Mr. D. Maluchnik for assistance in preparation of photographic documentation. We also thank Dr. J. Kubiak for critically reading the manuscript.

REFERENCES

Baker, S. M., Plug, A. W., Prolla, T. A., Bronner, C. E., Harris, A. C., Yao, X., Christie, D. M., Monell, C., Arnheim, N., Bradley, A., Ashley, T., and Liskay, R. M. (1996). Involvement of mouse *Mlh1* in DNA mismatch repair and meiotic crossing over. *Nat. Genet.* **13,** 336–342.

Blow, J. J., and Laskey, R. A. (1988). A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* **332,** 546–548.

Bornslaeger, E. A., Mattei, P. M., and Schultz, R. M. (1988). Protein phosphorylation in meiotically competent and incompetent mouse oocytes. *Mol. Reprod. Dev.* **1,** 19–25.

Borsuk, E., Szöllösi, M. S., Besombes, D., and Debey, P. (1996). Fusion with activated mouse oocytes modulates the transcriptional activity of introduced somatic cell nuclei. *Exp. Cell Res.* **225,** 93–101.

Bouniol, C., Nguyen, E., and Debey, P. (1995). Endogenous transcription occurs at the 1-cell stage in the mouse embryo. *Exp. Cell Res.* **218,** 57–62.

Bouniol-Baly, C., Hamraoui, L., Guibert, J., Beaujean, N., Szöllösi, M. S., and Debey, P. (1999). Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes. *Biol. Reprod.* **60,** 580–587.

Bouniol-Baly, C., Nguyen, E., Besombes, D., and Debey, P. (1997). Dynamic organization of DNA replication in one-cell mouse embryos: Relationship to transcriptional activation. *Exp. Cell Res.* **236,** 201–211.

Chesnel, F., and Eppig, J. J. (1995). Synthesis and accumulation of $p34^{cdc2}$ and cyclin B in mouse oocytes during acquisition of competence to resume meiosis. *Mol. Reprod. Dev.* **40,** 503–508.

Coverly, D., Wilkinson, H. R., and Downes, C. S. (1996). A protein kinase-dependent block to reinitiation of DNA replication in G2 phase in mammalian cells. *Exp. Cell Res.* **225,** 294–300.

Coverly, D., Wilkinson, H. R., Madine, M. A., Mills, A. D., and Laskey, R. A. (1998). Protein kinase inhibition in G2 causes mammalian Mcm proteins to reassociate with chromatin and restores ability to replicate. *Exp. Cell Res.* **238,** 63–69.

Cuthbertson, K. S. R. (1983). Parthenogenetic activation of mouse oocytes *in vitro* with ethanol and benzyl alcohol. *J. Exp. Zool.* **226,** 311–314.

Cuthbertson, K. S. R., Whittingham, D. G., and Cobbold, P. H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. *Nature* **294,** 754–757.

Dekel, N. (1995). Molecular control of meiosis. *Trends Endocrinol. Metab.* **6,** 165–169.

DePamphilis, M. L. (1998). Initiation of DNA replication in eukaryotic chromosomes. *J. Cell. Biochem. Suppl.* **30/31,** 8–17.

deVantery, C., Stutz, A., Vassalli, J. D., and Schorderet-Slatkine, S. (1997). Acquisition of meiotic competence in growing mouse oocytes is controlled at both translational and posttranslational levels. *Dev. Biol.* **187,** 43–54.

Fang, J., and Benbow, R. M. (1996). Nuclear proteins of quiescent *Xenopus laevis* cells inhibit DNA replication in intact and permeabilized nuclei. *J. Cell Biol.* **133,** 955–969.

Ferreira, J., and Carmo-Fonseca, M. (1997). Genome replication in early mouse embryos follows a defined temporal and spatial order. *J. Cell Sci.* **110,** 889–897.

Flaggs, G., Plug, A. W., Dunks, K. M., Mundt, K. E., Ford, J. C., Quiggle, M. R., Taylor, E. M., Westphal, C. H., Ashley, T., Hoekstra, M. F., and Carr, A. M. (1997). Atm-dependent interactions of a mammalian Chk1 homolog with meiotic chromosomes. *Curr. Biol.* **7,** 977–986.

Fulka, J., Jr., First, N. L., Lee, C., Fulka, J., and Moor, R. M. (1997). Induction of DNA replication in germinal vesicles and in nuclei formed in maturing oocytes by 6-DMAP treatment. *Zygote* **5,** 213–217.

Fulka, J., Jr., Horská, M., Moor, R. M., Fulka, J., and Kanka, J. (1996). Oocyte-specific modulation of female pronuclear development in mice. *Dev. Biol.* **178,** 1–12.

Fulton, B. P., and Whittingham, D. G. (1978). Activation of mammalian oocytes by intracellular injection of calcium. *Nature* **273,** 149–151.

Furuno, N., Nishizawa, M., Okazaki, K., Tanaka, H., Iwashita, J., Nakajo, N., Ogawa, Y., and Sagata, N. (1994). Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. *EMBO J.* **13,** 2399–2410.

Hartwell, L. H., and Weinert, T. A. (1989). Checkpoints: Controls that ensure the order of cell cycle events. *Science* **246,** 629–634.

Hua, X. H., and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc 6, but dependent on cdk2. *J. Cell Biol.* **140,** 271–281.

Leonhardt, H., and Cardoso, M. C. (1995). Targeting and association of proteins with functional domains in the nucleus: The insoluble solution. *Int. Rev. Cytol.* **162B,** 303–335.

Lima-de-Faria, A., and Borum, K. (1962). The period of DNA synthesis prior to meiosis in the mouse. *J. Cell Biol.* **14,** 381–388.

Mahbubani, H. M., Chong, J. P. J., Chevalier, S., Thömmes, P., and Blow, J. J. (1997). Cell cycle regulation of the replication licensing system: Involvement of cdk-dependent inhibitor. *J. Cell Biol.* **136,** 125–135.

Moore, G. D., Ayabe, T., Kopf, G. S., and Schultz, R. M. (1996). Temporal patterns of gene expression of G1–S cyclins and cdks during the first and second meiotic cell cycles in mouse embryos. *Mol. Reprod. Dev.* **45,** 264–275.

Moses, M. J., Dresser, M. E., and Poorman, P. A. (1984). Composition and role of the synaptonemal complex. *Symp. Soc. Exp. Biol.* **38,** 245–270.

Nakajo, N., Oe, T., Uto, K., and Sagata, N. (1999). Involvement of Chk1 kinase in prophase I arrest of *Xenopus* oocytes. *Dev. Biol.* **207,** 432–444.

Nurse, P. (1997). Regulation of the eukaryotic cell cycle. *Eur. J. Cancer* **33,** 1002–1004.

Pasero, P., and Gasser, S. M. (1998). New systems for replicating DNA in vitro. *Curr. Opin. Cell. Biol.* **10,** 304–310.

Peters, H., Levy, E., and Crone, M. (1962). Deoxyribonucleic acid synthesis in oocytes of mouse embryos. *Nature* **195,** 915–916.

Płusa, B., Ciemerych, M. A., Borsuk, E., and Tarkowski, A. K. (1997). Transcription and DNA replication of sperm nuclei introduced into blastomeres of 2-cell mouse embryos. *Zygote* **5,** 289–299.

Rao, P. N., and Johnson, R. T. (1970). Mammalian cell fusion. I. Studies on the regulation of DNA synthesis and mitosis. *Nature* **225,** 159–164.

Rowles, A., Tada, S., and Blow, J. J. (1999). Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. *J. Cell Sci.* **112,** 2011–2018.

Szöllösi, D., Czołowska, R., Borsuk, E., Szöllösi, M. S., and Debey, P. (1998). Nuclear envelope removal/maintenance determines the structural and functional remodelling of embryonic red blood cell nuclei in activated mouse oocytes. *Zygote* **6,** 65–73.

Szöllösi, M. S., Debey, P., Szöllösi, D., Rime, H., and Vautier, D. (1991). Chromatin behaviour under influence of puromycin and 6-DMAP at different stages of mouse oocyte maturation. *Chromosoma* **100,** 339–354.

Severova, E. L., Samoshkina, N. A., and Dyban, A. P. (1989). A study of the first cycle of DNA replication in parthenogenetic mouse embryos. *Ontogenez* **20,** 516–524. [In Russian]

Tarkowski, A. K., Witkowska, A., and Opas, J. (1977). Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. *J. Embryol. Exp. Morphol.* **41,** 47–64.

Tarkowski, A. K., and Wróblewska, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *J. Embryol. Exp. Morphol.* **18,** 155–180.

Thömmes, P., and Blow, J. J. (1997). The DNA replication licensing system. *In* "Cancer Surveys," Vol. 29, "Checkpoint Controls and Cancer," pp. 75–90. Imperial Cancer Research Fund, Cold Spring Harbor.

Usui, T., Yoshida, M., Abe, K., Osada, H., Isono, K., and Beppu, T. (1991). Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-252a. *J. Cell Biol.* **115,** 1275–1282.

Zhao, J., and Benbow, R. M. (1994). Inhibition of DNA replication in cell-free extracts of *Xenopus laevis* eggs by extracts of *Xenopus laevis* oocytes. *Dev. Biol.* **164,** 52–62.

Received for publication December 13, 1999
Revised April 4, 2000
Accepted April 11, 2000